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Additional inventors are being named on the ________ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Formation of Membrane Attack Complex on Circulating Immune Complexes

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☑ Application Data Sheet. See 37 CFR 1.76

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[Page 1 of 2]

Respectfully submitted,

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SPECIFICATION

[Electronic Version 1.2.8]

Formation of Membrane Attack Complex on Circulating Immune Complexes

Cross Reference to Related Applications

Field of Search 436/501, 506, 507, 512, 536, 539, 548, 821, 828 435/7References CitedU.S. PATENT DOCUMENTS4,960,712Theofilopoulos et al.4,978,611Hosoda et al.5,698,449Baumann et al.6,258,549 B1 Auer et al.FOREIGN PATENT DOCUMENTS20,010,801Chauhan et al (WO)

Background of Invention

[0001] Complement pathway is made of more than 35 plasma proteins that play key role in host defense against microbial infections and continuous clearance of apoptotic debris. The formation of an antigen-antibody complex (immune complex) is the principal mechanism of complement activation. Complement play key role in the antigen presentation and production of antibody responses by B cells. The activation of complement proteins can mainly occur by three major pathways, classical, alternate and mannose binding lectin pathway. These each pathway lead to formation of C3 convertase that cleaves the C3 complement protein to generate C3a, an anaphylotoxin and C3b that participates in formation of C5 convertase. At C5 convertase level the entire three pathways converge to activate the C5 molecule that results in formation of C5b and C5a another potent anaphylotoxin. The C5b forms the substrate for formation of a complex by associating with the complement protein C6, C7, C8 and up to sixteen molecules of C9 to form the terminal complement complex (TCC) also termed as membrane attack complex (MAC) or simply C5b-9. The MAC formation occurs once the complement protein C5 is cleaved into C5a and C5b. It is generally known that once the C5 convertase split the C5 to C5a and C5b, C5b then associate with the C6 and C7 to form C5b-7, this complex then inserts into cell membrane. Once the C5b-7 inserts into cell wall, it then acts as substrate for addition of the remaining components of MAC complex namely C8 and C9 on the cell membrane. The activation of complement components by CIC, the subsequent formation of MAC on CIC and the transfer to cell membrane can inflict a reversible cell damage or cell lysis. The amount of MAC transferred to cell membrane determines the fate of cell whether it goes into apoptosis

or necrosis. The sublytic dose of MAC on cell wall triggers multiple signaling pathways and initiates pleitropic responses.

[0002] Complement plays a key role in regulating the innate and adoptive immune responses. The activation of complement pathway and subsequent generation of C5a and MAC, TCC or C5b-9 by polymerization of terminal components of the complement are the key mediators of complement induced signaling pathways and inflammatory responses. In the present invention we for the first time demonstrate the presence of C5 and C5b-9 complexes on the CIC and demonstrate that the CIC act as the substrate for the formation of MAC and there subsequent transfer to the cell membrane. We also show that the complement split products present on CIC are non-covalently linked to CIC. Also the CIC carrying the higher levels complement proteins C3, C4, C5, and C5b-9 are present in patients in serum with higher disease activity. Chemokine production by alveolar macrophages in presence of C5a or C5b-9 was reported to be significantly higher after treatment with IgG circulating immune complexes (CIC). It was noted that for production of MIP-2, CINC, MCP-1 and MIP-1 α by C5a and or C5b-9 in intrapulmonary environment, the presence of IgG containing CIC was essential. [Czermak et al, Am. J. Pathol. 154(5):1513 (1999)] The data accumulated over years have supported the notion that the formation C5 convertase leading to generation of C5a and C5b-9 is a major component of chronic inflammatory process associated with atherosclerosis [Yasojima et al, Am. J. Pathol. 158(3):1039(2001)], myocardial infarction and myocardial ischemia and reperfusion injury [Vaveka et al, Circulation 97:2259(1998); Afanasyeva et al, Am. J. Pathol. 161(2):351(2002)].

[0003] In lieu the significance of the activation of C5 molecule and subsequent generation of C5a and C5b-9 it is critical to understand the mechanisms and localization of C5a and C5b-9 during activation. The complement activation occurs via three main pathways that converge at the C5 convertase step. The C5 convertase activity to split the C5 molecule into C5a and C5b is provided by C3bBb3b, the alternative pathway convertase and C4b2aC3b, the classical pathway convertase. The C5a fragment is released and the two-chain C5b fragment provides the substrate for the formation of the C5b-9. The larger fragment C5b associates with C6 and C7, which forms an amphiphillic entity, which is capable of inserting itself in the cell membrane The C8 then joins the complex and unwinds in the membrane. Finally up to sixteen molecules of C9 join to form C5b-9 complex.

[0004] The presence of split products of C5 or C5b-9 complex associated with CIC has never been reported earlier. The common belief is that the cleavage of C5 occurs in fluid phase thus generating C5b and C5a. The association of C5b to C6 and C7 occurs in the fluid phase leading to the formation of C5b-7 complex, which inserts itself into the plasma membrane. The fate of soluble C5b-7 complex is also determined by the soluble complement inactivating factors such as S protein, Clustrin or vitronectin present in

soluble phase. The binding of C5b-7 to these proteins inhibits the insertion of the complex into the cell membrane. In a model of rat alveolar macrophage model it was reported that soluble MAC which has little ability to bind to cell surface, did not enhance lung injury after intrapulmonary deposition of IgG CIC [Czermak et al, Am. J. Pathol. 154(5):1513(1999),].

[0005] The recent work on the C5b-9 molecular complex has shown the importance of the C5b-9 molecular complex in the apoptosis, necrosis and pro-inflammatory pathways. The C5b-9 is the principal mediator of injury induced by antibodies experimentally directed against glomerular cell membrane. C5b-9 in sublytic concentration enhances the production of endothelial intercellular adhesion molecule-1 (ICAM-1) and E selectin, while directly inducing production of interleukin 8 (IL-8) and monocyte chemo attractant protein-1 (MCP-1) [Kilgore et al, J. Immunol. 155:1434(1995)]. It has been also reported that the C5b-9 activates transcription factors NF-κ B and AP-1 resulting in the production of IL-6 in human smooth muscle cells [Viedt et al, FASEB J. 14:2370(2000].

[0006] In podocytes the number of C5b-9 inserted into cell membranes determines whether cell undergoes necrosis. Formation of sublytic C5b-9 on the cell membrane results in release of calcium, activation of specific signaling pathways, increase in growth factor production, increased oxidants and proteases [Couser W.G. J. Am. Soc. Nephrol. 1:13(1990); Cybulsky et al, Am J. Pathol. 155:1701(1999)]. The sublytic dose of C5b-9 on cell membrane activated cell cycle related genes i.e. p53, p21, growth arrest DNA damage- 45 (GADD45), checkpoint kinase-1 (CHK-1) and CHK-2. The extra cellular signal-regulated kinase (ERK) is critical pathway involved in regulating these cell cycle related proteins following C5b-9 induced DNA damage [Pippin et al, J. Clin. Invest. 111:877 (2003)].

[0007] Assembly of C5b-9 on cells of the arterial wall induces cell lysis. The sublytic assembly of C5b-9 on smooth muscle cells and endothelial cells induce cell activation and proliferation. Sublytic assembly of C5b-9 on the plasma membrane activates p 38 MAPK, Janus kinase (JAK) 1, signal transducer and activator (STAT) 3 and STAT 4 in endothelial cells. [Niculescu et al, J. Immunol. 158:4405(1997)].

[0008] In the passive Heymannn nephritis model of membranous nephropathy, the assembly of C5b-9 induces glomerular epithelial cell (GEC) injury and proteinuria that is partially mediated via production of eicosanoids. The sublytic formation of C5b-9 induces phosphorylation of epidermal growth factor receptor (EGF-R), Neu, fibroblast growth factor receptor-2 and hepatocyte growth factor receptor. The phosphorylation of tyrosine (204) of ERK-2 as well as free [(3) H] arachidonic acid (AA) and prostaglandin E (2) was stimulated by the formation of C5b-9 on cell membrane. It has been concluded that C5b-9, induces trans-activation of receptor tyrosine kinases, in association with

ERK2 activation, AA release and PGE (2) production in cultured GEC and glomerulonephritis. [Cybulsky et al, Am. J. Pathol. 155:1701(1999)].

[0009] Complement activation and membrane assembly of sublytic C5b-9 play an important role in inflammation by promoting cell proliferation and by rescuing cell apoptosis. The sublytic concentrations of C5b-9, increases Ca^{+} influx, activates phospholipases, increase level of diacylglycerol (DAG) & ceramide, activates protein kinase C (PKC) and generates arachidonic acid. In post-mitotic cells such as oligodendrocytes (OLG) and skeletal muscles, C5b-9 reverses the differentiation of the cell phenotype. [Shirazi et al, J. Neurochem. 48:271(1987)]. Sublytic C5b-9 also induces proto-oncogenes, activates the cell cycle, and enhances survival by inhibiting apoptosis [Rus et al, J. Immunol. 156:4892(1996); Halperin et al, J. Clin. Invest. 91:1974(1993)]. In OLG loss of differentiation due to C5b-9 attack was associated with the activation of proto-oncogene c-jun, c-fos, and junD and induction of AP1 DNA binding activity. C5b-9 is the most potent ERK1 inducer. ERK1 activation was preceded by activation of membrane-associated Gi, Ras and Raf-1 then activation of cytoplasmic MAPK/ERK kinase (MEK) 1. Trimeric Gi protein was also activated [Niculescu et al, J. Immunol. 158:4405(1997); Niculescu et al, J. Biol. Chem. 269:4417(1994)].

[0010] The complement proteins also play key role in the neurodegenerative diseases like Alzheimer. In several models it has been demonstrated that C5a via anti-apoptotic provides neuroprotection [Mukherejee et al, J. Neurochem. 77:43 (2001)]. Thus it may be of significant importance to specifically block the formation of MAC on the CIC thereby regulating the transfer of MAC in significant doses to avoid the necrosis of the neuronal tissue. The tissue damage by complement and the lytic doses of the MAC leading to tissue necrosis and inflammatory responses has been the key mechanism in diseases such as lung injury, injury to podocytes, cardiomyopathies, myasthenia gravis, multiple sclerosis, cerebral lupus erythematosus, Guilain-Barre syndrome, Alzheimer"s disease, lupus nephritis, membranous nephritis, membrane proliferative glomerulonephritis, rheumatoid arthritis, systemic lupus erythematosus, Behcet"s syndrome, juvenile idiopathic arthritis, Sjogren"s syndrome, atheroma, thyreoiditis, infertility, vasculitis, post bypass syndrome, tissue incompatible transplantation.

Summary of Invention

[0011] Disclosed in the invention is the formation of Membrane Attack Complex (MAC), Terminal Complement Complex (TCC) or C5b-9 on Circulating Immune Complexes (CIC) and the potential application of measurement complement products and MAC and its components on CIC as diagnostic marker and blockage of MAC formation on CIC for therapy in diseases. For the first time we report that CIC acts as substrate for formation of MAC and there subsequent transfer on to cell membrane.

[0012] The present invention describes the presence of non-covalently associated complement proteins with the CIC. The invention describes for the first time the formation of MAC, on the CIC. The experimental data presented in the invention describes that the MAC and components of early complement activation present on CIC are not linked to CIC via covalent linkage. The invention describes that during plasmapheresis the complement split products and MAC present on CIC are released from the CIC changing the nature of CIC from pathogenic to non-pathogenic. The invention describes that the measurement of complement products and MAC on CIC as a tool for monitoring the disease activity and therapy status in diseases mediated by CIC and complement. The present invention utilizes, in its broadest sense, the modifications of complement linked to CIC provide beneficial effect in complement and CIC as a means to mediated diseases.

Brief Description of Drawings

[0013] In the drawings forming the disclosure of this invention:

[0014] **FIG.1** illustrates the binding of AHG (aggregated human gamma-globulin) used as immune complex model. A standard curve for binding of AHG was generated in ELISA technique. A linear binding was achieved using the concentration of 2.34, 4.68, 9.37, 18.75, 37.5, 75, 150 and 300 μg/ml of AHG. The appropriate dilutions were made in PBS/Tween 20.

[0015] **FIG.2** illustrates the presence of CIC composed of various immunoglobulin isotypes (IgG, IgA and IgM) in patient"s plasma suffering from SLE and RA.

[0016] **FIG.3** demonstrate the presence of activated complement components C1q, C3, C4, C5 and C5b-9 in the CIC present bound to CIC in patient plasma from autoimmune diseases.

[0017] **FIG.4** illustrates the standard curves for (a) C1q, (b) C3, (c) C4, (d) C5 and (e) IgG-CIC used for determining the concentration of the respective components present within the CIC from the patient plasma sample.

[0018] **FIG.5** demonstrates the effect of 25 mM EDTA on the binding of activated complement protein C5 and C5b-9 to CIC. The samples subjected to estimation of C5 and C5b-9 was mixed with appropriate concentration of EDTA so as to bring the final concentration to 25 mM at pH 7.5. Two parallel sets of paired samples from the same patient, one treated with EDTA and other control group without EDTA were subjected to C5 and C5b-9 estimation. As demonstrated in the Figure the presence of EDTA decreased the presence of complement proteins in CIC. It was concluded from this

experiment that the complement proteins in these CIC were not linked with them due to covalent linkage.

[0019] **FIG.6** demonstrates the effect of plasmapheresis on complement binding to CIC. Sequential samples from patient"s undergone kidney and heart transplant and treated with plasmapheresis to achieve beneficial clinical results were obtained for analysis. These samples were then analyzed for the amount of CIC composed with various immunoglobulin isotypes IgG, and IgM. The samples were subjected to measurement of complement proteins C1q, C3, C4, C5 and C5b-9 bound to CIC pre and post plasmapheresis. As demonstrated in the figure the complement levels bound to CIC decreased significantly with the plasmapheresis. This provided an added experimental proof for in vivo interaction of complement with CIC is not mediated by covalent linkage.

[0020] **FIG.7** demonstrates the 2D SDS-PAGE (Two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis) of CIC purified from rheumatoid arthritis patient. The CIC were purified using affinity resin made by coupling the receptor protein isolated as per patent (PCT/US02/24301). The receptor protein was coupled to NHS activated Sepharose beads (NHS- Sepharose 4B FF, Pharmacia AB, Piscataway, NJ, US). The resin was packed in a volume of 1.5 ml in a sterile disposable polystyrene column. The patient plasma was allowed to interact with the resin and after washing the non-bound material, the CIC were eluted by lowering the pH to 3.5 with Glycine-HCl buffer. The purified CIC were reduced using DTT and 2ME and the proteins were first separated based on charge in the first dimension using IEF and based on molecular weight on the second dimension by polyacryamide gel electrophoresis. The CIC components were recognized by comparing the gel image to 2D SDS-PAGE image of human serum proteins in the NBRF protein database. The CIC purified with the affinity column displayed the presence of heavy chain of globulins both γ and μ heavy chains and both light K and λ light chains thereby confirming the identity of CIC. The association of acute phase serum protein CRP was also identified associated in the CIC.

Detailed Description

[0021] Using receptors isolated (a detailed method for receptor preparation is described in detail patent ref. 20, 010, 801 Chauhan et al,) from cell lines, we for the first time have demonstrated that the assembly of C5B-9 does occur on the CIC. The second important finding we report in the study is that complement bound to the CIC is not attached covalently. Thus CIC may act as the substrate for formation of C5B-9 and subsequent transfer to the target cell surface. The presence of higher amount of C5b-9 on CIC leads to the transfer of lytic doses of C5B-9 to cell surface leading to necrosis, the sublytic doses of C5b-9 lead to cell activation, proliferation, apoptosis and a number of other cellular events. Biochemical or biological molecule restricting the formation of MAC on CIC will provide a therapeutic target as the reducing the amount of

MAC from lytic dose to sublytic will be beneficial approach for treating complement and CIC mediated injuries.

[0022] In our experiments we isolated receptors from a lymphoblastoid cell line that bind to CIC composed with IgG, IgM and IgA isotypes of immunoglobulin. These complexes are composed of antigen, antibody and other acute phase reactants from the patient plasma such as complement proteins, C reactive protein and SAP. Subsequently after capturing the CIC on solid phase coated with the receptor, we analyzed the CIC for their composition. In order to analyze the composition of the CIC from disease patients we used the ELISA based assays. To demonstrate the presence of complement proteins in the CIC we specifically used antibodies directed to complement proteins to demonstrate their presence in the CIC. There was no external additive added to the interaction thus no outside interference from other proteins was involved in these reaction. A previously titrated secondary antibody-HRP conjugate was used to measure the binding of isotype specific CIC. For measuring the complement proteins bound to CIC the antiserum specific to such complement proteins was allowed to interact with the complex bound to solid phase of the ELISA plates. Subsequently using secondary antibody-HRP conjugate directed to this serum was used for measuring the amount of complement proteins bound within these complexes. Purified proteins and in vitro formed complexes were used as standards in separate wells to quantitate constituents of these CIC.

[0023] In our analysis utilizing serum samples from patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) we have demonstrated that during the disease activity the CIC demonstrate significant variation in there composition with respect to the presence of immunoglobulin isotypes IgG, IgA and IgM. In addition to the composition of the CIC isotypes the sera from these patients also demonstrated significant levels of complement proteins C1q, C3 C4, C5 and C5b-9 associated with the CIC. It is known that opsonization of CIC with these complement proteins is necessary for clearance of apoptotic debris generated during the normal physiology as well as during the infection. Any defect in clearance of CIC, or the excessive formation of CIC during infection is a major pathologic event resulting in development of auto immune disorders [Walport MJ, Arthritis Res. 4(suppl 3):S279 (2002)]. For the first time we were able to demonstrate the presence of C5 component of complement and reactivity to C5b-9 bound to the CIC. However using the antibodies directed towards C5a we were not able to detect any reactivity thus concluding that C5a after cleavage from the C5b falls into the soluble phase and does not form the part of the complex.

[0024] This is the finding we expected from these assays as is generally known that CIC binds and activates the classical component of the complement pathway. The split product of complement C3b and C4b bind to proteins via the formation of thiol ester bond that gets exposed via activation. The product of C4A gene binds to CIC by

formation of amide bond while the product of C4B gene binds via carboxyl group through esterification. In our experiments first time we demonstrate that the major portion of the activated complement components present on CIC are not covalently attached but only a small portion of the complement appears to be linked via covalent linkage. To confirm this fact we performed two experiments. In one experiment we analyzed the effect of EDTA on the complement proteins bound to CIC. In this experiment we demonstrated that by treating the serum sample with ethylene diamine tetra acetic acid (EDTA), the complement proteins bound to the CIC is released. We analyzed serum samples that had been previously tested for the presence of CIC and complement in presence and absence of EDTA. The serum samples from five patients were diluted 1:20 with PBS containing 0.05% Tween 20. One set of samples was treated with 25 mM EDTA that was included in the diluent buffer. The inclusion of EDTA dramatically reduced the binding of complement proteinsC3, C4, C5 and C5b-9 in the CIC. This demonstrated that inclusion of 25 mM EDTA in the PBS as a diluent and subsequent in the incubation step during the binding while did not affect the amount of immunoglobulin isotype amounts however eliminated the presence of the complement proteins associated to CIC.

[0025] In another experiment the patient serum samples collected from the pre and post plasmapheresis were analyzed for the presence of complement proteins bound to CIC, we demonstrated that the plasmapheresis affected the binding of complement proteins to CIC. A dramatic drop from 40 to 90% was demonstrated with various complement proteins bound to CIC providing the beneficial affect of the therapy. This experiment reaffirmed our in vitro observation, there by demonstrating that even in vitro complement proteins are associated to CIC via non-covalent linkage

[0026] The nature of CIC was established by selectively isolating CIC from patient with RA and SLE. The CIC was purified using an affinity resin developed by coupling the receptor preparation with Sepharose FF 4B (Pharmacia, Piscataway NJ). The affinity resin was then utilized for capture of CIC from the RA and SLE patients. The individual components of CIC were displayed on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and the individual components were recognized by Western Blotting. The identity of individual immunoglobulin chain was established using μ and γ heavy chain specific antibodies. The identity of the components of the CIC was also established by subjecting the CIC to 2D SDS-PAGE analysis. Using the comparative analysis of the results from the NBRF protein database we established the presence of IgM and IgG heavy chains as well as both kappa and lambda light chains in the captured CIC.

[0027] The measurement of CIC composed with various immunoglobulin isotypes and the activated complement component C1q, C2, C3, C4, C5, and C5b-9 bound to CIC is a useful indicator. The modulation of the complement on CIC by a chemical,

biochemical, peptide or biological will useful in the treatment of the disease as: ♣Renal Diseases◆Anti-Glomerular Basement Membrane Disease◆Renal Vasculitis: Focal Necrotizing GlomerulonephritisoRapidly progressive glomerulonephritisoWegener"s granulomatosus (WG)oMicroscopic polyangitisoIdiopathic RPGN◆Focal Segmental Glomerulosclerosis◆Systemic Lupus Erythematosus◆Anti-Glomerular Basement Membrane Disease♣**Neurological Disease**◆Eaton-Lambert Syndrome◆Guillain-Barre" syndrome◆Amyotrophic Lateral Sclerosis◆Myasthenia Gravis◆Inflammatory Polyneuropathy◆Multiple Sclerosis◆Alzheimer ♣**Hematological Disease**◆Mycelia and Cryoglobulinemia◆Thrombotic Thrombocytopenic Purpura◆Idiopathic Thrombocytopenic Purpura◆Allaoantibodies in Hematologic Disease♣**Rheumatologic Disease**◆Rheumatoid Arthritis◆Rheumatoid Vasculitis◆Scleroderma◆Dermator◆Early stages of Scleroderma◆Dermatomyosistis◆Polymyosistis◆Sjogren"s syndrome◆Behcet"s disease ♣**Other Disease**◆Pemphigus Vulguris associated to antibodies to squamous epithelium◆Bullous pemphigoid associated to antibodies to dermal basement◆Cardiovascular Disease:◆Myocardial Infarction◆Cardiomyopathies◆Ischemia reperfusion injury◆Transplant◆Neoplastic DiseasesExample One: The example describes the development and use of ELISA based assays for measurement of components of CIC i.e. Antibody isotypes, IgG (IgG1, IgG2, IgG3, IgG4), IgA, IgM; complement proteins, C1q, C3, C4, C5 and C5b-9; other acute phase proteins associated within the CIC.

[0028] **Details on performing the assay for measuring the components of CIC:**

[0029] (1)Purified receptor preparation the proteins binding specifically to CIC via Fc portion were dissolved in an alkaline buffer (0.1M sodium carbonate pH 9.6).

[0030] (2)The alkaline solution with receptor preparation was placed in contact with the plate at 4°C for 12 to 24 hours.

[0031] (3)The coating was removed form the plate and plate washed three times with a solution of sodium chloride (0.15M), buffered by sodium and potassium phosphate (0.01M, pH 7.2 to 7.4) (PBS) to remove unbound receptors.

[0032] (4)Thereafter to block free sites the plate was placed in contact with 100 µl of 1% BSA dissolved in PBS containing 0.05% Tween-20.

[0033] (5)The blocking solution was removed and plates washed three times with PBS fortified with 0.05% of Tween-20 (v/v).

[0034] (6)The sera from patients were diluted properly with a solution of PBS prior to testing. In this example we diluted the sera 10 volumes and 20 volumes of PBS.

[0035] (7)A total of 0.1 ml aliquots of diluted sera were placed into appropriately designated wells. For this example duplicate determinations were performed for each specimen and average values were used for calculations.

[0036] (8)The plates were kept at 37°C in humid container for two hours.

[0037] (9)The plates were washed again with PBS/Tween-20.

[0038] (10) The plates were filled with 100 µl of appropriate anti serum for measurements (anti-human IgG-HRP, anti-human IgM-HRP, anti-human IgA-HRP, anti-human C3, anti-human C4, anti-human C1q, anti-human C5 and anti-human C5b-9). The plates were incubated at room temperature for sixty minutes.

[0039] (11)The plates were washed again and the plates that received the HRP conjugates were developed for HRP enzyme activity. Otherwise the wells in plates without conjugated antiserum were filled species, specific anti-HRP conjugate and further incubated for sixty minutes at room temperature.

[0040] (12)After washing each plate well in the plate was assayed for horseradish peroxidase activity by addition of 100 µl substrate buffer (TMB substrate).

[0041] (13)The reaction was monitored for the development of color and at appropriate color density the reaction was terminated by addition of 25 µl of 2.5 M H_2-SO_4.

[0042] (14)Optical Density was measured and plotted against the standards concentrations and the linear equation was used to obtain the quantity of components in the CIC (Figure No. 4).

[0043] Example Two:

[0044] **Purification and Analysis of CIC on 2D SDS-PAGE:**(1)The receptor protein binding was conjugated to NHS-activated (n-hydroxyl succinamide) sepharose 4 B (Pharmacia, Piscataway, USA).

[0045] (2)The free sites on the resin were blocked with excess of 1M Tris-HCl pH 7.5.

[0046] (3)The resin was washed with PBS to remove unbound Tris-HCl.

[0047] (4)In a column with one ml of resin a total of 1.5 ml of patient plasma was placed in contact with the receptor bound resin.

[0048] (5)The plasma was allowed to flow under gravity and fifteen times PBS was allowed to flow into the column to remove unbound plasma proteins.

[0049] (6)The bound CIC were eluted with low pH buffer (Glycine-HCl, 0.1M, pH 3.5).

[0050] (7)Captured CIC were concentrated to a final volume of 300 µl.

[0051] (8)Twenty micro-liters of the purified CIC proteins were mixed with IEF renaturing solution consisting of 8M Urea, Bridge 58, NP40, 2ME, β-Octylglucoside.

[0052] (9)The sample was mixed properly and applied to 7 mm IPG strips (Bio-Rad, Hercules, CA). The strips were left for 16 hours at room temperature thereafter the IPG strips were subjected to isoelectric focusing on pH 3.5 to 10 IPG strip in accordance with manufacturer recommendation.

[0053] (10)A total amount of 10,000 Volt-hours were applied during the IEF.

[0054] (11)After the isoelectric focusing the strips were removed from the IEF cell and drained of excess mineral oil and incubated with buffer containing 8M Urea, 0.375 M of Tris-HCl buffer pH 8.8, 20% Glycerol, 100mM DTT for 15 minute with constant shaking.

[0055] (12)After the first incubation the IEF strips were incubated for another fifteen minute at room temperature in a buffer with composition similar to earlier buffer containing 125 mg of Iodoacteamide per 10 ml of buffer.

[0056] (13)Thereafter the IPG strips were overlaid for second dimension run on 4 to 12% SDS-PAGE NuPAGE gel (Invitrogen, Carlsbad, CA).

[0057] (14)The electrophoresis was carried out in MOPS buffer at 170 volts for two hours. The gels were then fixed in acetic acid and ethanol fixative.

[0058] (15)The gels were stained with silver stain. The comparative analysis of the, CIC were done utilizing the 2D protein database from EMBO to establish the identity of globulin heavy and light chains.

Claims

[c1]

What is claimed is:

1.Presence of complement split products C5 and formation of membrane attack complex (C5b-9) on CIC.

[c2]

2.Blocking the formation of MAC on CIC to obtain beneficial therapeutic effect in patients suffering from complement and CIC mediated diseases.

[c3]

3.Screening or designing a useful composition for blocking the formation of MAC on CIC as in claim 2.

[c4]

4.The usefulness of monitoring the formation of MAC and other split products of C5 on CIC as claimed in claim 1, from the serum, plasma, CSF and other bodily fluids in diseases associated with complement and CIC pathogenesis, including but not limited to autoimmune diseases, cardiovascular diseases, neurodegenerative diseases, infectious disease and oncological diseases.

[c5]

5.The demonstration of presence of non-covalent linked complement split products C1q, C3, C4, C5 and MAC on CIC.

[c6]

6.Blocking the association of C1q, C3, C4, C5 and MAC on CIC as claimed n claim 5 to obtain beneficial therapeutic effect in patients suffering from complement and CIC mediated diseases.

[c7]

7.Screening or designing a useful composition for blocking the formation of MAC on CIC as claimed in claim 6.

[c8]

8.The usefulness of monitoring the complement split products C1q, C3, C4, C5 and MAC on CIC as claimed in claim 5, for diagnosis and monitoring the disease activity in diseases pathogenicity associated with complement and CIC pathogenesis including but not limited to autoimmune diseases, cardiovascular diseases, neurodegenerative diseases, infectious disease and oncological diseases.

[c9]

9.Development of a process for quantitative measurement for the presence of complement C5 and C5b-9 as claimed in claim 1,associated with CIC. The said process comprising the following steps:

a.Providing a test device comprising the receptor preparation in solid phase (prepared in accordance with the process in patent 20, 010, 801) as a capture reagent for CIC.

[c10]

b.Establishing selected working range for said immunoassay within said ranges of composition of CIC.

[c11]

c.Constructing a standard assay curve by plotting relative degree of immunochemical binding of said CIC components to the test device.

[c12]

d.Interacting a fixed concentration of the immunospecific conjugate of said substances, the composition of complexes resulting from said immunological substances and immunospecific conjugate being within the selected working limits of step.

[c13]

e.Providing a test system comprising of said test device, said immunospecific conjugate, said immunological substances, the amount of said immunospecific conjugate being substantially equivalent to said fixed concentration of immunospecific conjugate, and the amount of said immunospecifically determinable substance being appropriate to produce a known degree of immunochemical binding corresponding to a pre determined point on said standard curve, thereby enabling quantitative assaying of complement C5 and C5b-9 present on CIC.

[c14]

10.Usefulness in monitoring the complement proteins C1q, C3, C4, C5 and C5b-9 bound via non covalent to CIC and CIC as claimed in claim 5 for early diagnosis and monitoring of disease activity in humans suffering with complement and CIC mediated injuries including but not limited to autoimmune, cardiovascular, neurodegenerative disorders, oncological diseases and infectious disease

.

[c15]

11.Develop a process for measurement of complement proteins C1q, C3, C4, C5 and C5b-9 as in claim 8 from the plasma and other bodily fluids of patients suffering from including but not limited to autoimmune, cardiovascular, neurodegenerative disorders, oncological diseases and infectious disease. Said process comprises:

a.Providing a test device comprising the receptor preparation in solid phase (prepared in accordance with the process in patent 20, 010, 801(WO)) as a capture reagent for CIC.

[c16]

b.Establishing selected working range for said immunoassay within said ranges for complement proteins CIC.

[c17]

c.Constructing a standard assay curve by plotting relative degree of immunochemical binding of said complement components to the test device.

[c18]

d.Interacting a fixed concentration of the immunospecific conjugate directed to complement proteins and immunospecific conjugate being within the selected working limits of step.

[c19]

e.Providing a test system comprising of said test device, said immunospecific conjugate, said immunological substances, the amount of said immunospecific conjugate being substantially equivalent to said fixed concentration of immunospecific conjugate, and the amount of said immunospecifically determinable substance being appropriate to produce a known degree of immunochemical binding corresponding to a pre determined point on said standard curve, thereby enabling quantitative assaying of complement C1q, C3, C4, C5 and C5b-9 present on CIC.

[c20]

12.Developing a process for quantitation of immunoglobulin isotype composition of CIC or antigens bound within CIC using ELISA based on receptor based capture mechanism, said process comprises:

a.Placing the receptor on solid phase of ELISA plates, micro beads or any other suitable surface.

[c21]

b.Attaching the biotin or any other form of detection tag on the antigen or antibody.

[c22]

c.Mixing the tagged antigen or antibody with the patient plasma, patient serum, sinuovial fluid, cerebrospinal fluid (CSF) or any other bodily fluid.

[c23]

d.Placing the mixture from 10c in contact with receptor on the solid surface 6a.

[c24]

e.Washing the unbound components with buffers.

[c25]

f.Quantitating the tagged antigen or antibody with a reagent such as Avidin-Hoarse Radish Peroxidase and color development reagents.

[c26]

13.Development of a process as claimed in claim 3 for screening the composition of a blocking agent for the formation of MAC and deposition of C5 on CIC.

[c27]

14.Development of a process as claimed in claim 8 for screening of the composition of a blocking agent for blocking the association of split products C1q, C3, C4, C5 and MAC to CIC as claimed in claim 6 to achieve beneficial therapeutic affects in complement and CIC mediated diseases.

[c28]

15.A process for screening the composition that targets blocking of complement activation or other components assembly in the CIC as said in claim 11 and 12, and modulate the binding of serum acute phase proteins bound to CIC, said process comprising

a.Attaching the receptor to solid phase or studying the interaction in the liquid phase, allowing the interaction of the CIC with the receptor in presence of complement proteins to activate complement deposition or other acute phase proteins on CIC.

[c29]

b.Placing the blocking composition during the activation of the complement on CIC or association of serum acute phase protein.

[c30]

c.The composition could be a chemical, biochemical, protein, peptide and monoclonal antibody prepared by any technique known to people in the art.

[c31]

d.Obtaining initial data indicating whether the formation of MAC and binding of complement C1q, C2, C3, C4, and C5 is inhibited on the CIC.

[c32]

e.Obtaining initial data indicating whether the serum acute phase proteins association with the CIC is inhibited.

[c33]

16.A process for determining the extent of blocking of complement activation on CIC, said process comprising of:

a.Attaching the receptor to solid phase or studying the interaction in the liquid phase, allowing the interaction of the receptor with the serum or plasma of patients suffering from complement and CIC mediated injuries to study activated complement deposited on CIC.

[c34]

b.Placing the blocking composition during the interaction of the receptor or disease plasma or serum.

[c35]

c.The composition could be a chemical, biochemical, protein, peptide and monoclonal antibody prepared by any technique known to people in the art.

[c36]

d.Obtaining initial data indicating whether the MAC or complement activation product C1q, C2, C3, C4 and C5 on CIC are reduced by composition in claim 7d on the CIC.

[c37]

e.Obtaining initial data whether the serum acute phase proteins associated with CIC are reduced by composition in claim 9e.

[c38]

17. The composition of claim 13 and or 14, wherein said composition is used for obtaining beneficial therapeutic outcome including but not limited to infections, cardiovascular diseases, neurodegenerative diseases, renal disease, rheumatologic diseases, neoplastic disease, and transplant in human patients.

[c39]

18.A method of reducing disease symptoms in an individual comprising: identifying an individual in need of reducing the symptoms due to increased complement fixation on CIC leading to inflammation and tissue necrosis by administering a composition from claim 13 and or 14.

[c40]

19.A process in accordance with claim 11 and 12 that further comprises contacting a receptor during interaction with CIC and complement with at least one of humanized monoclonal antibodies, active molecules, peptides and mimotopes and obtaining data indicative of whether the activation of complement has been inhibited.

[c41]

20.A process in accordance with claim 17 that additionally comprises inoculating patients or animals with the immune complex and composition, wherein the immune complex mediated immune responses are altered providing beneficial effect.

Abstract of Disclosure

[0059] The invention relates to demonstration of formation of membrane attack complex (MAC) and presence of complement protein C5 on the CIC and non-covalent association of activated complement products on circulating immune complexes (CIC). The invention relates to measurement of MAC and complement proteins C1q, C3, C4, C5 bound to CIC as marker of disease activity and pathogenicity for complement and CIC mediated diseases. The invention relates to modifications and blocking of non-covalent complement components, formation of MAC and association of C5 to CIC for therapeutic application in pathological disease with involvement of CIC and complement.

Figures

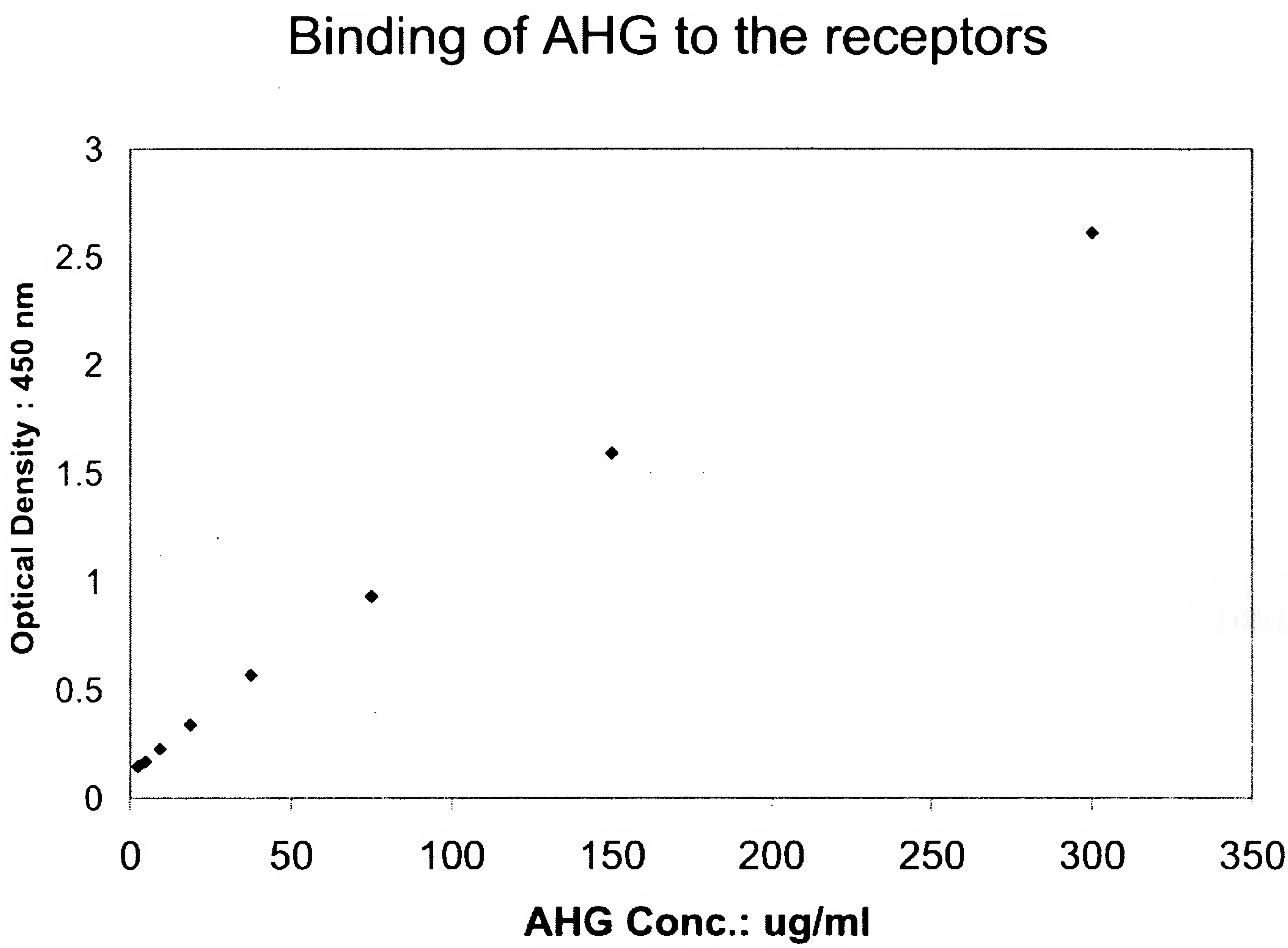
Binding of AHG to the receptors
Optical Density : 450 nm
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
300
350
AHG Conc.: ug/ml

Figure 1

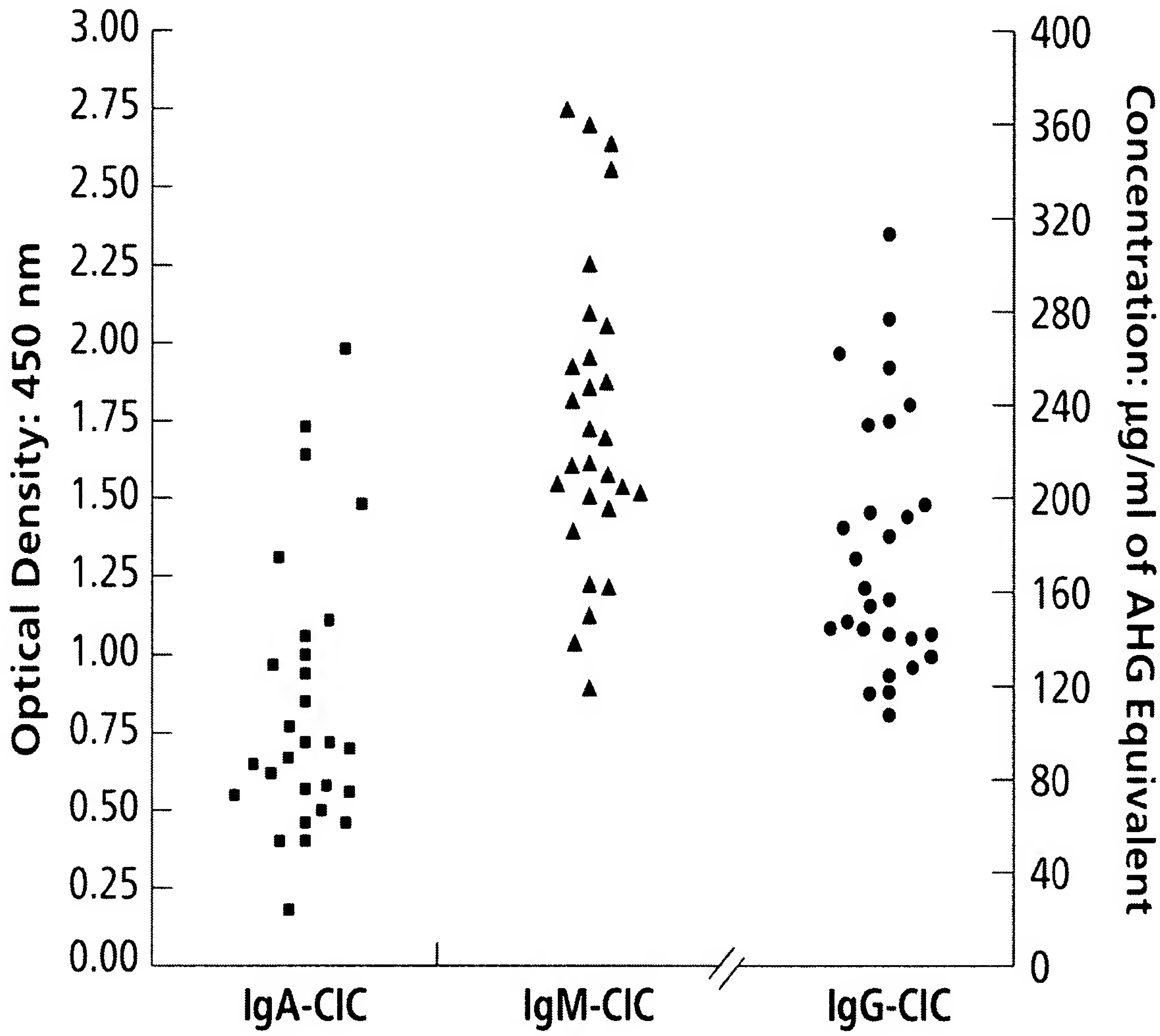
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Optical Density: 450 nm
400
360
320
280
240
200
160
120
80
40
0
Concentration: µg/ml of AHG Equivalent
IgA-CIC
IgM-CIC
IgG-CIC

Figure 2

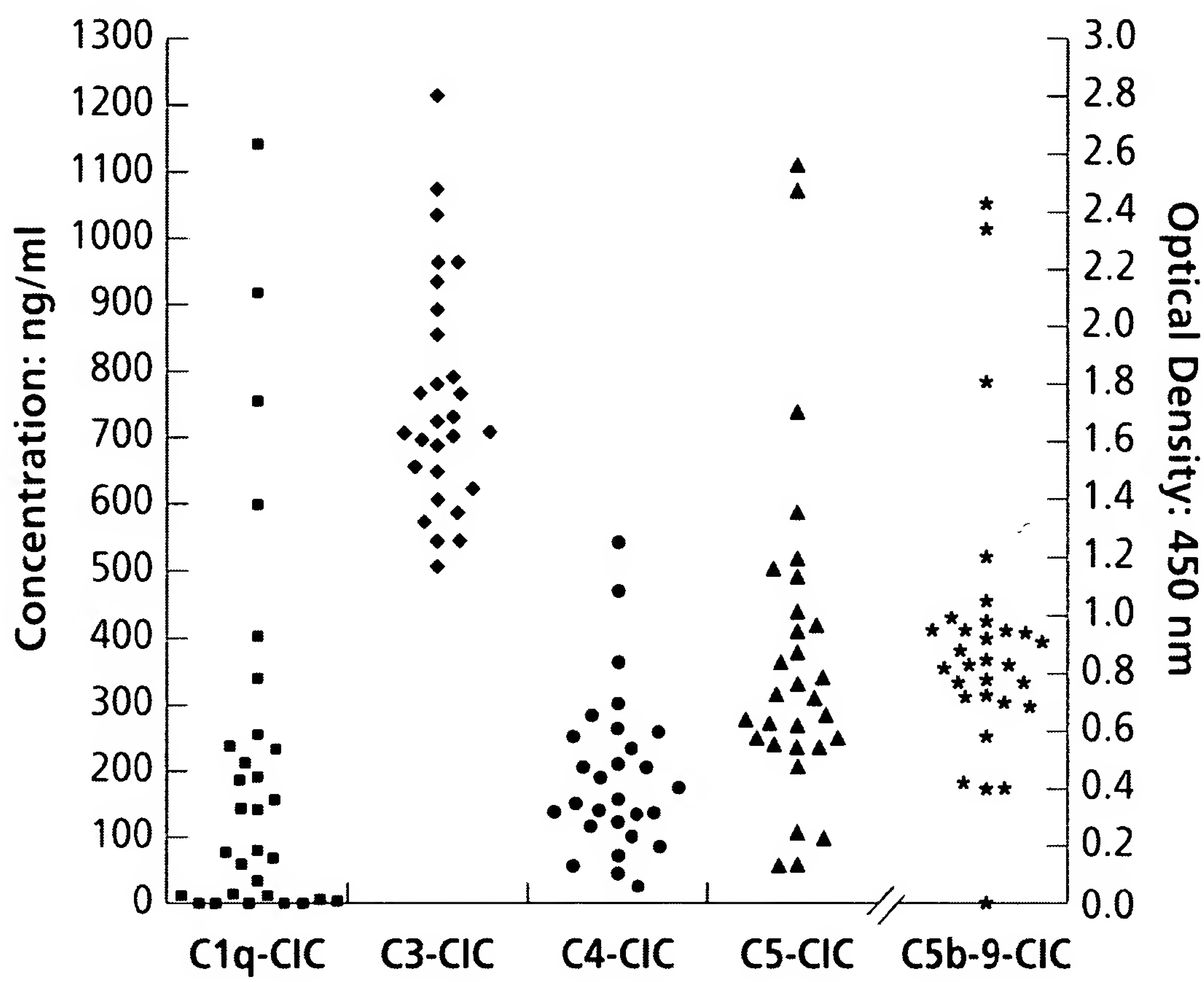
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Concentration: ng/ml
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density: 450 nm
C1q-CIC
C3-CIC
C4-CIC
C5-CIC
C5b-9-CIC

Figure 3

Formation of membrane Attack Complexes on CIC, Anil K. chauhan, 314-477-9592

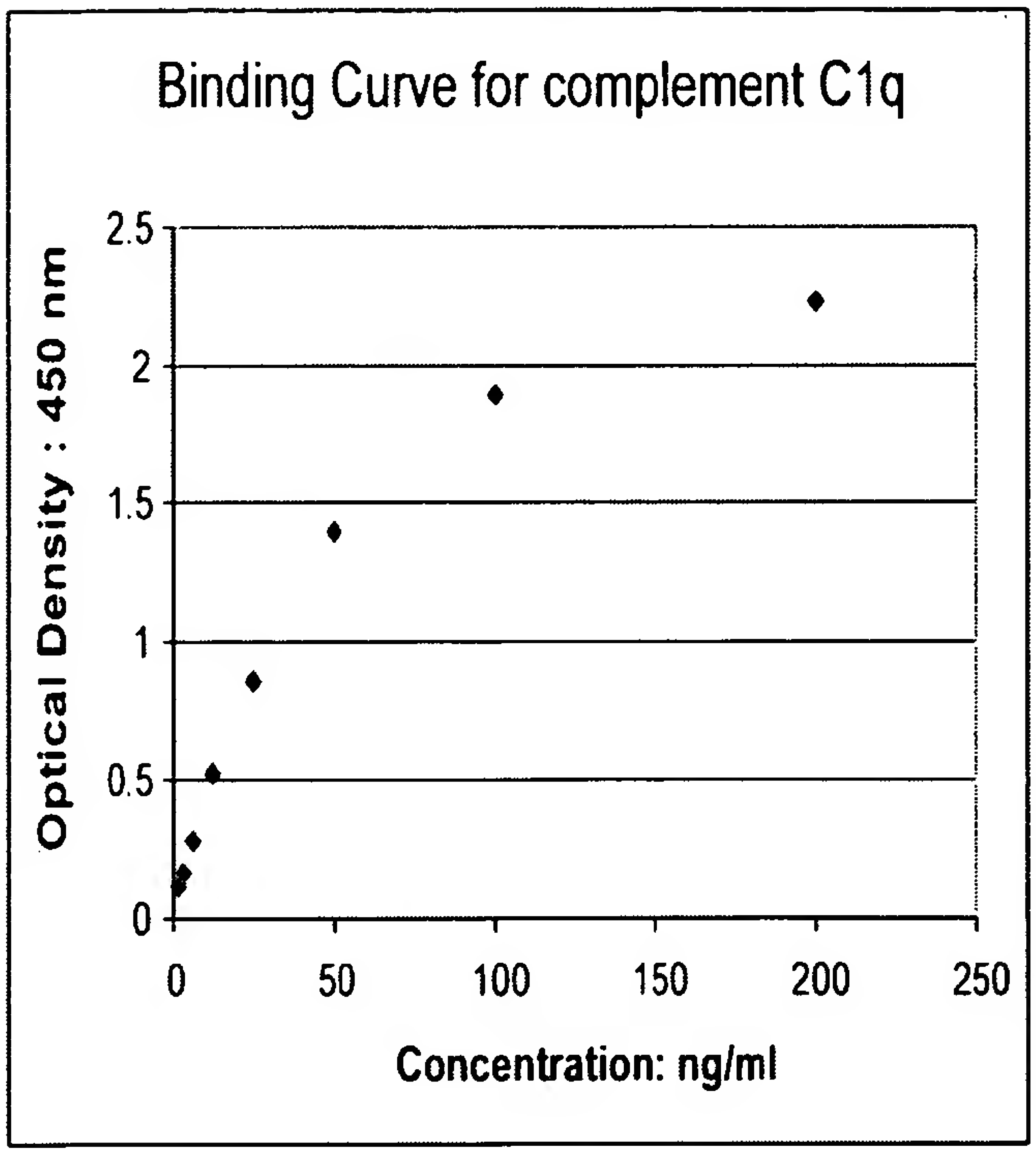

Figure 4a

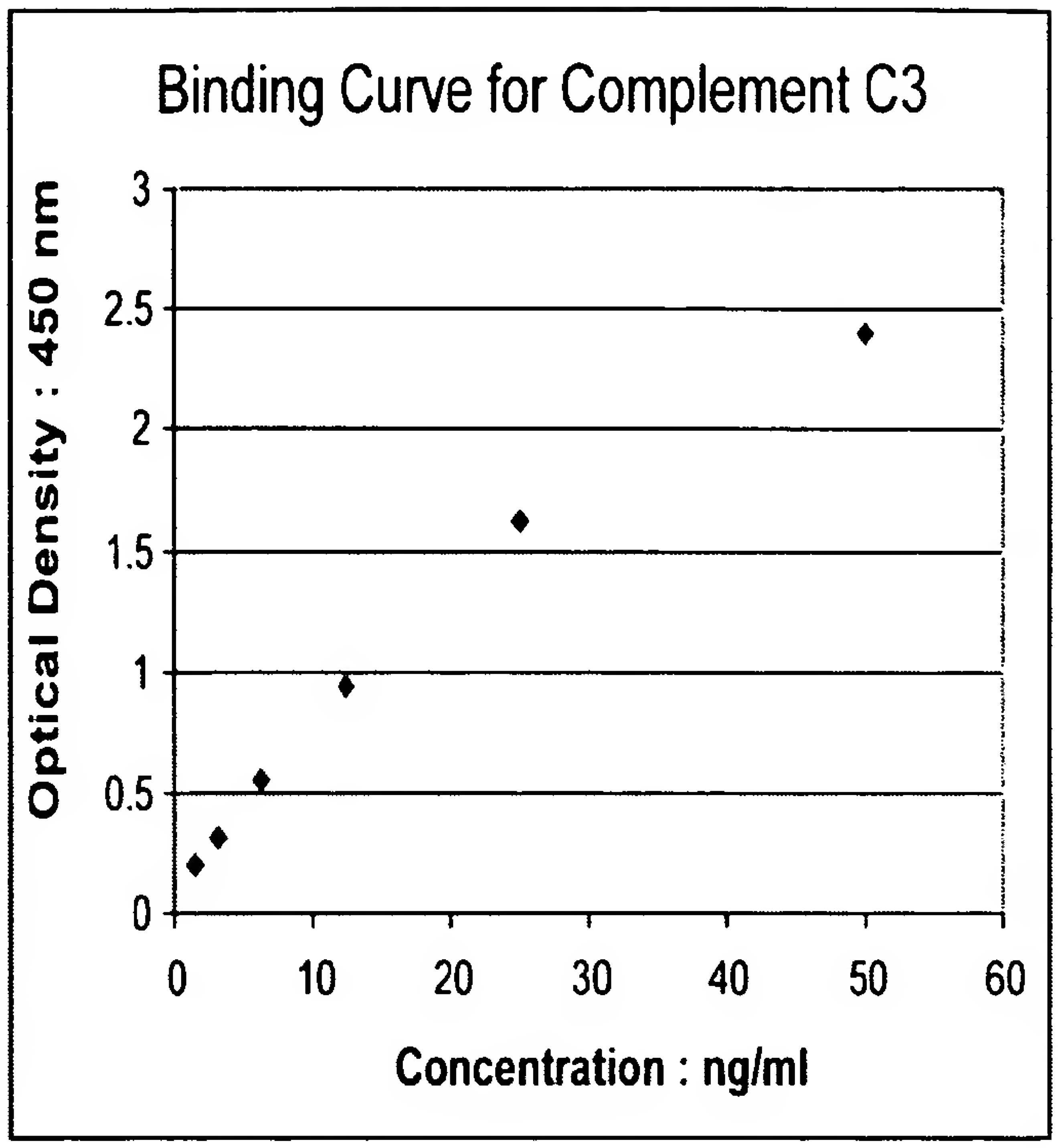

Figure 4b

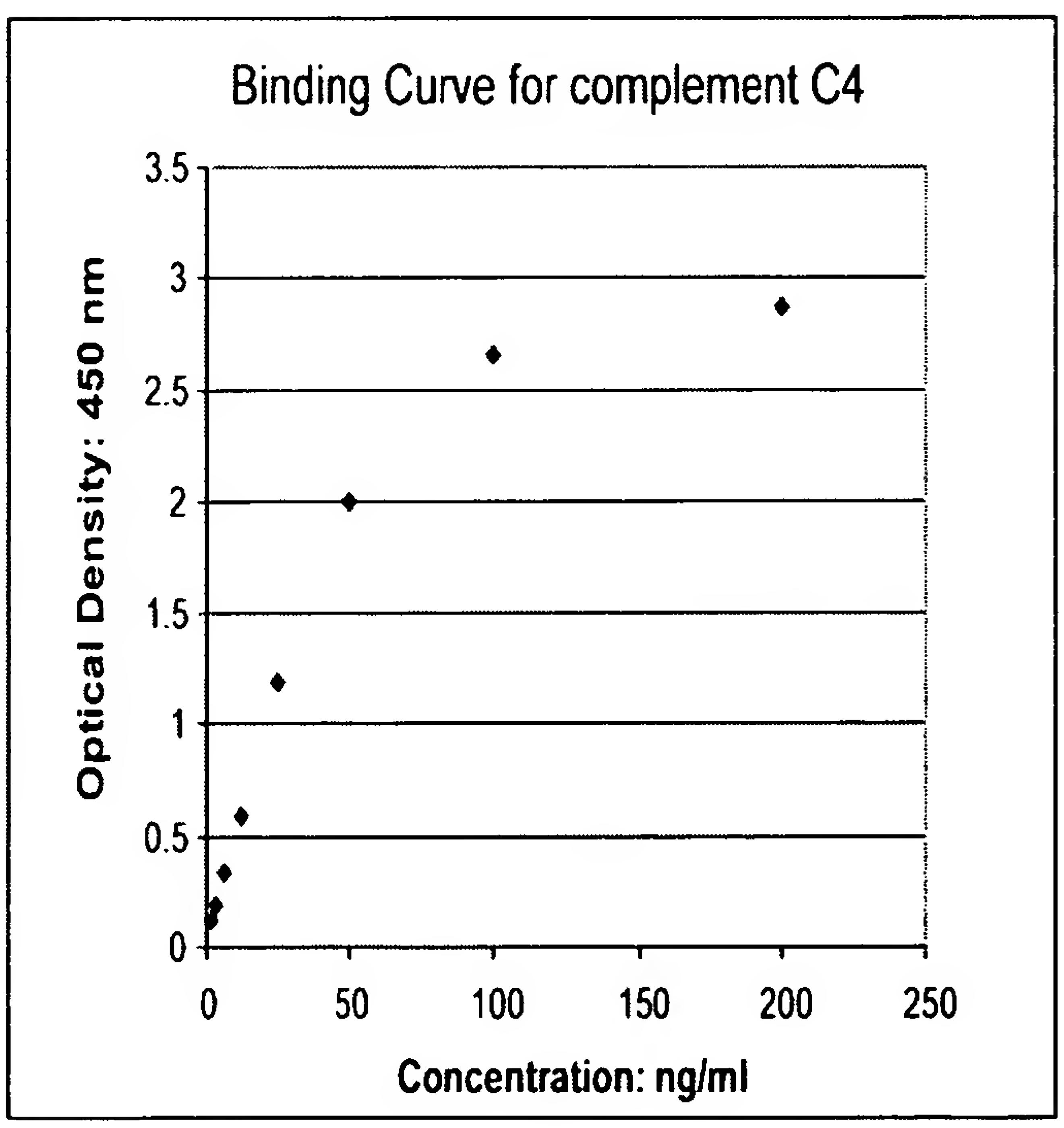

Figure 4c

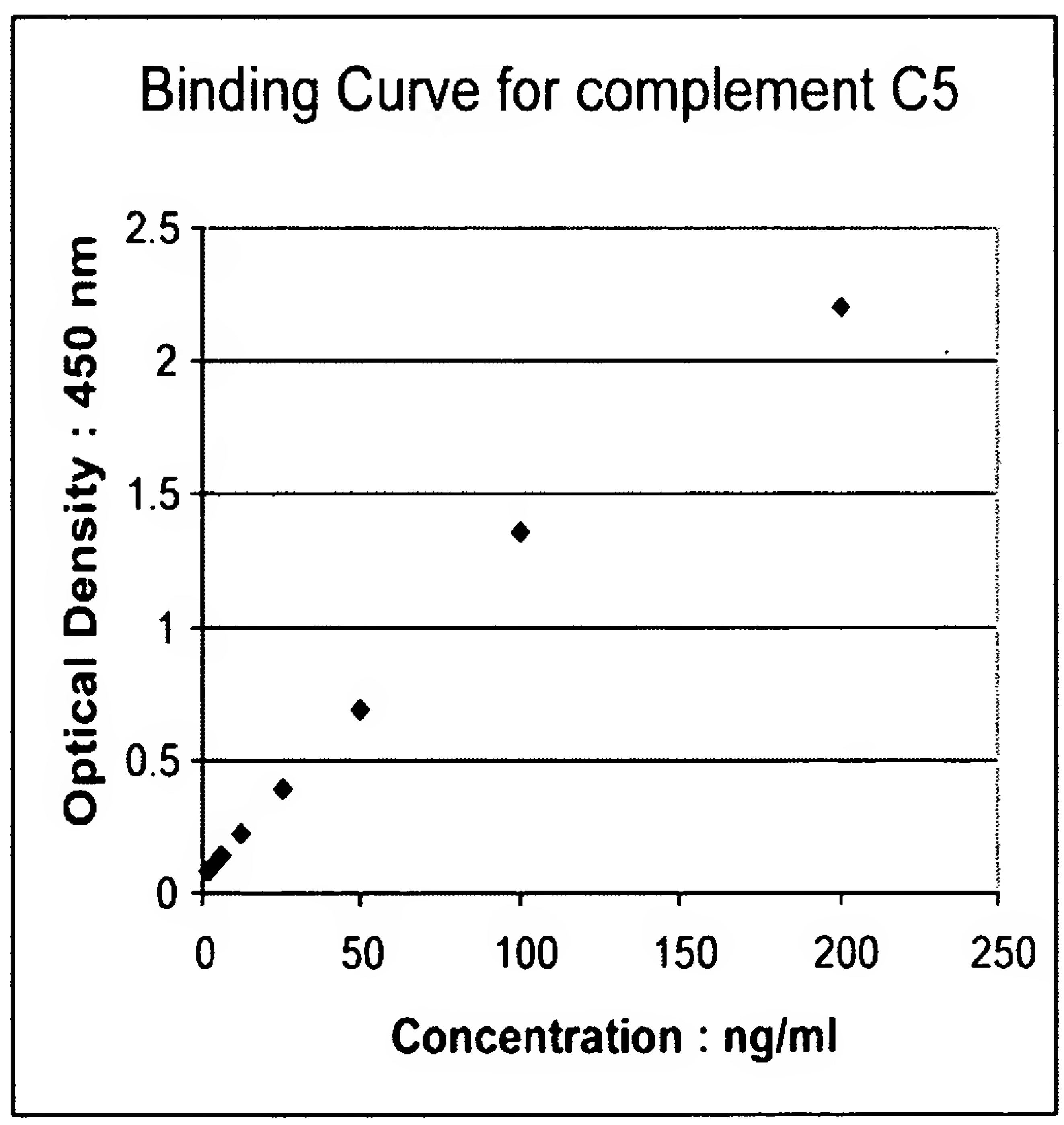

Figure 4d

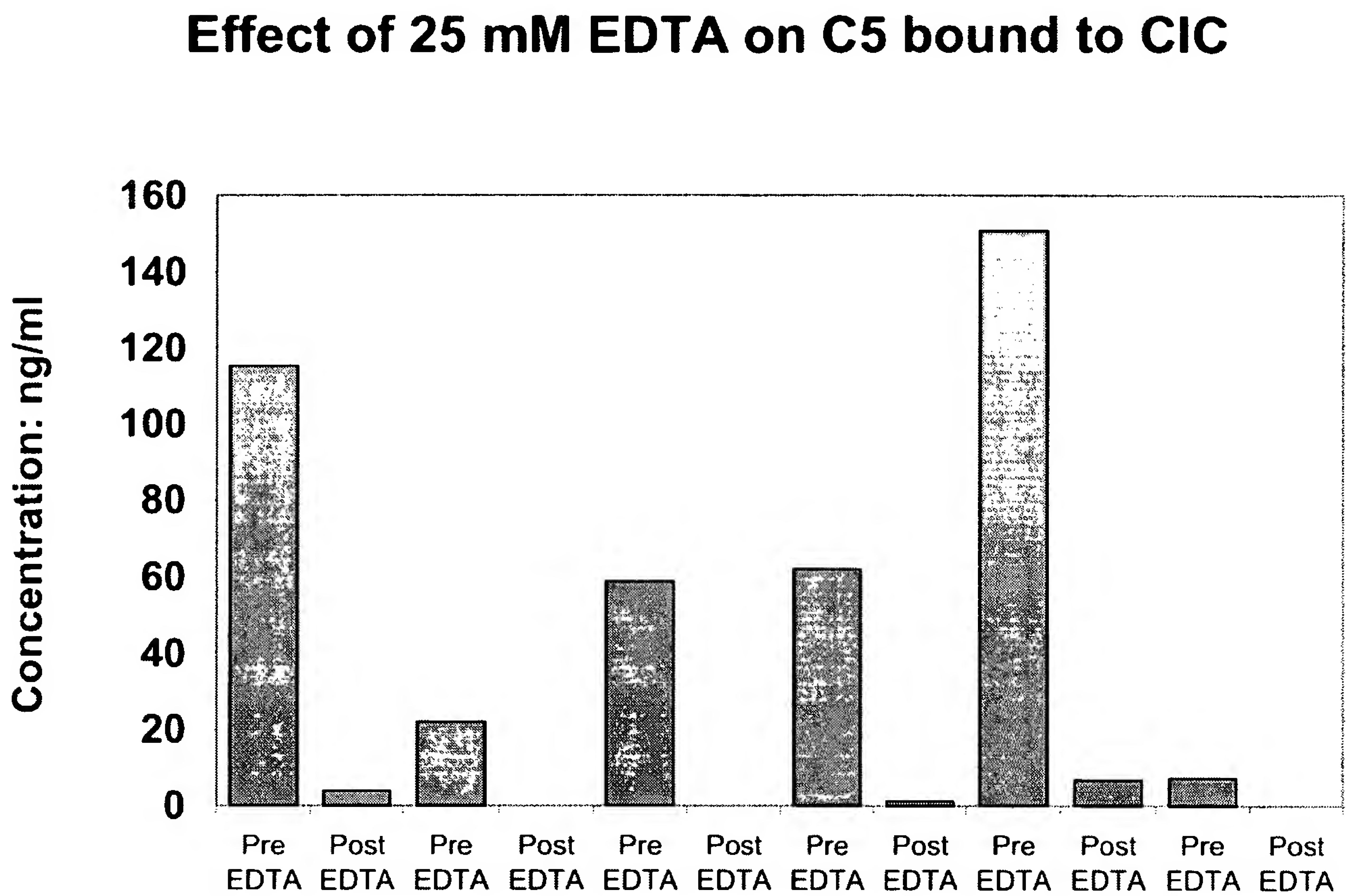

Figure 5a

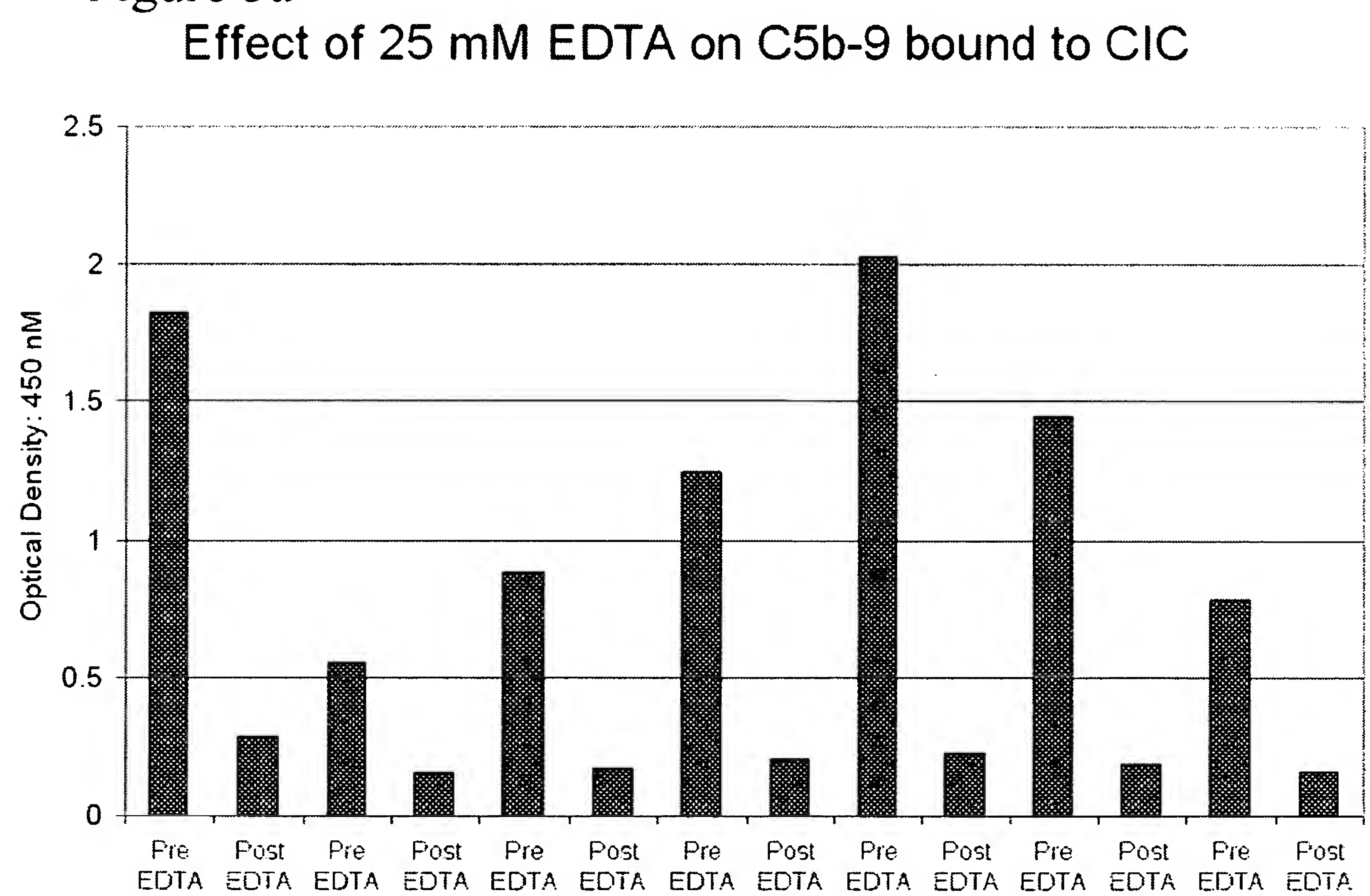

Figure 5b

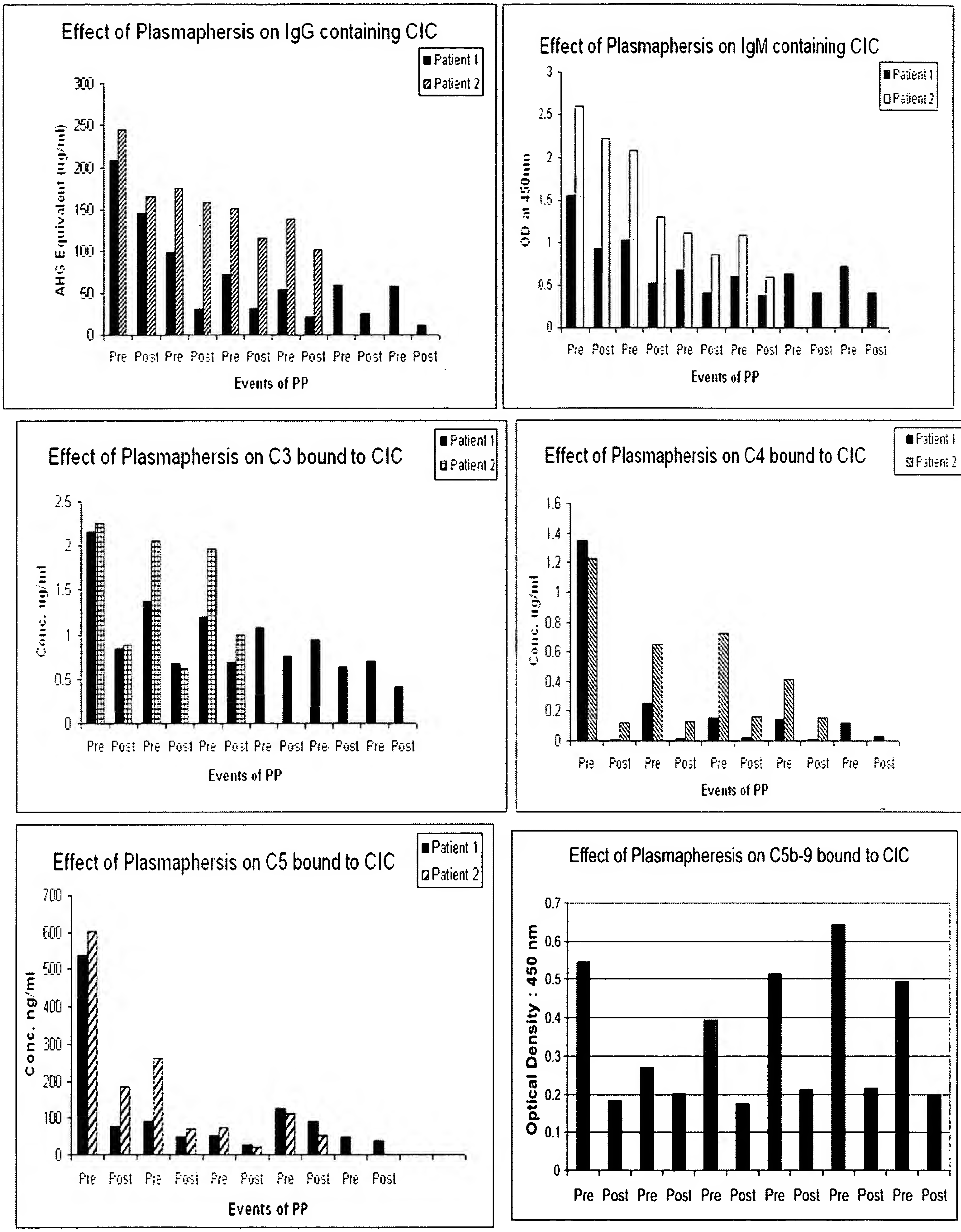
Effect of Plasmaphersis on IgG containing CIC
Patient 1
Patient 2
AHG Equivalent (ug/ml)
300
250
200
150
100
50
0
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post
Events of PP
Effect of Plasmaphersis on IgM containing CIC
Patient 1
Patient 2
OD at 450nm
3
2.5
2
1.5
1
0.5
0
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post
Events of PP
Effect of Plasmaphersis on C3 bound to CIC
Patient 1
Patient 2
Conc. ug/ml
2.5
2
1.5
1
0.5
0
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post
Events of PP
Effect of Plasmaphersis on C4 bound to CIC
Patient 1
Patient 2
Conc. ug/ml
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pre Post Pre Post Pre Post Pre Post Pre Post
Events of PP
Effect of Plasmaphersis on C5 bound to CIC
Patient 1
Patient 2
Conc. ng/ml
700
600
500
400
300
200
100
0
Pre Post Pre Post Pre Post Pre Post Pre Post
Events of PP
Effect of Plasmapheresis on C5b-9 bound to CIC
Optical Density : 450 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post

Figure 6

Formation of Membrane Attack complexes on CIC, Anil K. chauhan, 314-477-9592

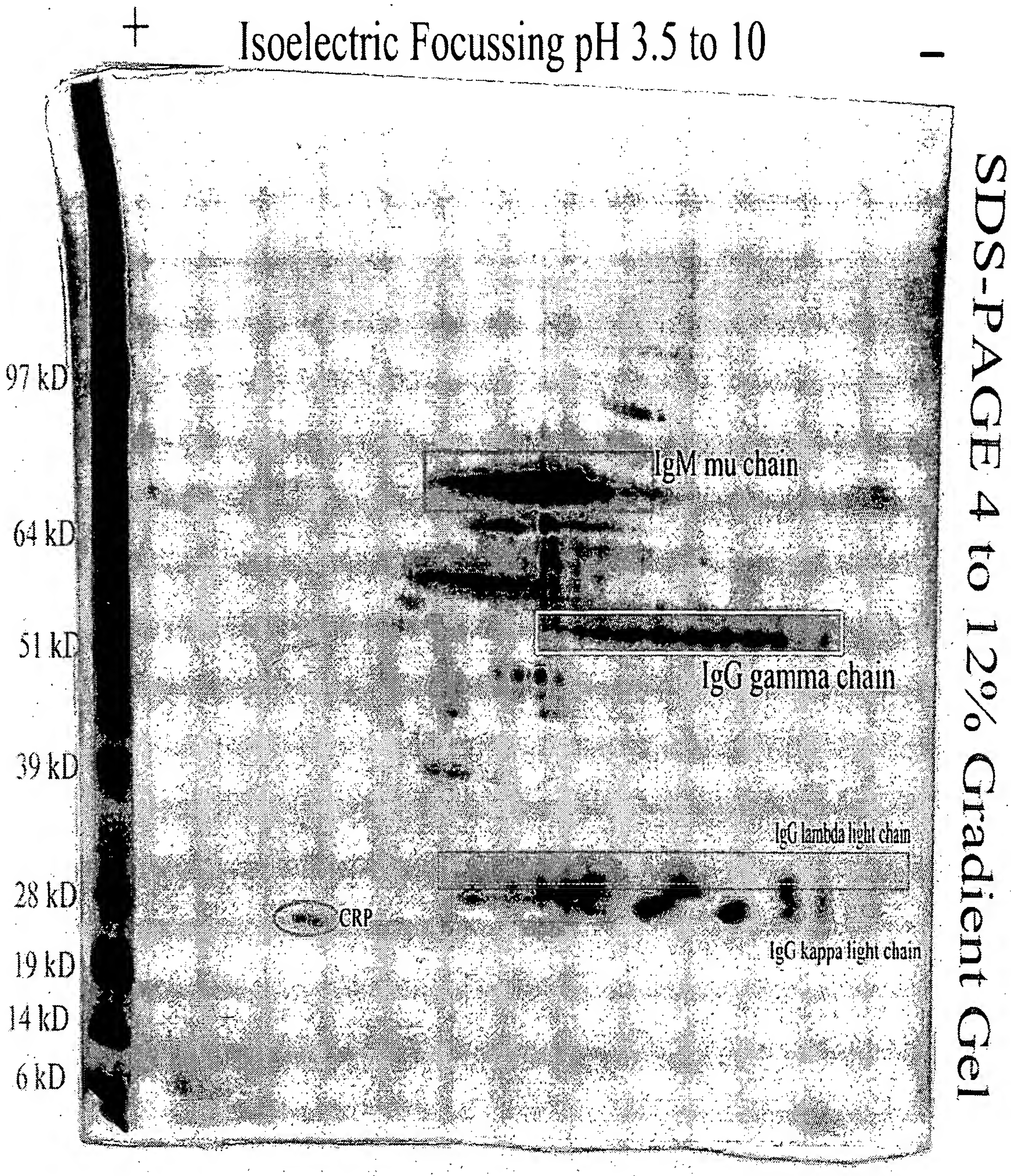

Figure 7

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Approved for use through 06/30/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Formation of Membrane Attack Complexes on Circulating Immune Complexes

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☑ The attached application, or

☐ Application No. ____________, filed on 01-28-04,

☐ as amended on ____________(if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Anil K. Chauhan

Signature: AKChauhan Citizen of: United States of America

Inventor two: ____________

Signature: ____________ Citizen of: ____________

Inventor three: ____________

Signature: ____________ Citizen of: ____________

Inventor four: ____________

Signature: ____________ Citizen of: ____________

☐ Additional inventors or a legal representative are being named on ____________ additional form(s) attached hereto.